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MODULATING EFFECTS OF NEUTRACEUTICALS ON ANTIOXIDANT ENZYMES IN AMMONIA INTOXICATION IN RATS

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ABSTRACT

Nutrition and lifestyle are well-defined modulators of chronic diseases. Poor dietary habits coupled with sedentary lifestyle contribute to today's compromised quality. It is becoming increasingly clear that nutrition can modulate the toxicity of environmental pollutants like ammonia. The study was designed to determine the possible protective effect of neutraceuticals against ammonium acetate induced oxidative stress. Experimental hyperammonemia was induced in adult male wistar rats (150-175g) by intraperitoneal injections of ammonium acetate (125mg/kg body weight). At the end of experimental duration serum and hepatic biomarkers were analyzed. The results revealed that ammonium acetate induced hyperammonemia, lipid peroxidation, and altered antioxidant enzymes in ammonium acetate treated group as compared with the normal group. On the other hand, neutraceuticals enriched diets reversed the biochemical indices indicating antihyperammonemic, hepatoprotective and antioxidant activities of neutraceuticals against ammonium acetate. Nutritional interventions rich in antioxidants may provide the means to develop primary prevention strategies of diseases associated with many environmental toxic insults.

KEYWORDS: Nutrition, Ammonia, Oxidative stress, Lipid Peroxidation, Neutraceuticals

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INTRODUCTION

Food sustains us and makes us realize our genetic potential. In those who eat to live, it goes to build and maintain functional capacity and physiological efficiency of various tissues, organs and systems. Food has also been implicated in making and breaking health. Of late this realization has started dawning on the physicians, policy planners and, even in the common human being. The significance of food has been highlighted by Greek physician Hippocrates some 2500 years ago who coined the phrase *Let food be thy medicine and medicine be thy food* (Jones, 1923).

Renewed interest in the prophylactic and therapeutic properties of food in today's times can be traced to the ever increasing occurrence of the so called life style diseases on one hand and to the inclination for awareness on disease prevention and health promotion. Studies indicate that these diseases originate in womb and are clinically manifested much later in life (Barker, 1998) The incidence of chronic or degenerative diseases has begun to become more frequent as a consequence of lopsided relationship of people with the environment in both developing countries as well as those in transitional stage of development. The environmental factors include life style factors namely dietary patterns and physical activity. The other factors contributing to their occurrence are smoking, stress, illicit drugs, exposure to radiations, environmental pollutants and toxicants. These factors together with genetic predisposition result in the initiation and progression of diseases.

There is clear evidence that exposure to environmental chemicals or pollutants can contribute to compromised health and the pathology of many age-related diseases (Delfino et al. 2005; Hennig et al. 2005; Needham et al. 2005). Most human exposure involving toxic chemicals or mixtures appear to originate from environmental and occupational sources (Centers for Disease Control and Prevention (CDC) 2005; Schafer and Kegley 2002). Uncontrolled hazardous waste sites and the ever-increasing use and accumulation of chemical pollutants viz. heavy metals, ammonia, polychlorinated biphenyls (PCB), persistent organic pollutants (POPs), are a major environmental and public health concern in many countries.

Ammonia; an irritant affects the skin, eyes and respiratory passages. It is extremely toxic when inhaled in concentrated vapors and repeated exposure may lead to bronchitis and pneumonia. It can cause chemical burns, cataracts and corneal damage, and has been shown to produce skin cancer. Disruptions to the ecosystem can result, with toxic effects to plants, animals and fish. The EPA lists ammonia as a toxic chemical on its Community Right-to-Know list. Found in a wide range of household cleaning products including glass cleaners, all-purpose cleaners, disinfectants and more, it is also produced endogenously by a catabolic product of protein and nitrogenous compounds that is formed in mammals and humans. At high levels, ammonia is neurotoxic, it affects the functions of the central nervous system, and leads to coma and death (Ellman, 1959). Hyperammonemia, caused by insufficient removal of ammonia in the liver (Wolheim, 1984) or portacaval shunting (Murthy et al. 2001), leads to an increase in ammonia level in the brain (Murthy et al. 2001), which is responsible for development of hepatic encephalopathy (Norenberg et al. 2004; Rama Rao et al. 2005). Ammonia intoxication impairs mitochondrial function (Wang et al. 1997), which could lead to decreased ATP synthesis and also to increased formation of free radicals (Shirwaikar et al. 2003). The major toxic effects of ammonia likely involve changes in cellular pH and the depletion of certain citric acid cycle intermediates, in particular α-ketoglutarate. It has been reported that sustained hyperammonemia in mice leads to increased lipid peroxidation in liver and brain, reflecting an oxidative stress condition (O'Conner et al., 1990).

Diet plays an important role in the prevention of chronic diseases. Plant foods are low in calories, rich in fibers, vitamins and minerals. Apart from these they also contain specific non nutrient metabolites or bioactive substances such as phenolics, thiols, carotenoids, tocopherols, glucosinolates that impart colour, flavour, other functional properties and above all various health benefits.

Plants synthesize these bioactive substances for their own benefit as protection against pests, mostly micro-organisms, bacteria and fungi, the ravages of free radicals and UV radiations. When consumed along with plant foods they get an access to our bodies and tissues where they perform the same protective roles and thus contribute to the reduction of chronic diseases. These metabolites have low potency as compared to pharmaceutical drugs, but since they are ingested regularly and in significant amounts as part of diet, they may have a noticeable long term physiological effect.

The present study was aimed at appraising oxidative stress potential of ammonium acetate and its modulation by natural neutraceuticals, tomatoes (lycopene), rice bran oil (oryzanol and tocotrienols), green tea (caetchins) and sugarcane wax (policosanol).

MATERIALS AND METHODS

Animal Care and Monitoring

Thirty six, healthy male albino rats of wistar strain, seven weeks old with an average weight of about 150-175 g

were procured from the small animal house of Chaudhary Charan Singh Haryana Agriculture University Hissar (CCSHAU), India. Animals were given the standard pellet diet (Hindustan Liver Ltd., India) and water *ad libitum* during acclimatization period of 1 week. The diet contained 20% protein, 5% protein, 5% fat and 5% fiber, 60% carbohydrates and 10% mixture of vitamins and minerals. They were housed individually in the polypropylene cages with sterilized wood chip bedding in a specific pathogen free animal house room under the constant environmental condition with 12 hour light and dark cycle, 22±1 ^{0}C temperature and 50 ±10% relative humidity. The study protocol was approved by Institutional Animal Ethics Committee (IAEC) of the University constituted as per the directions of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Neutraceuticals

Policosanol was generously provided by Biocon Ltd Banglore-560100,India; Lycored was a generous gift from Jagsonpal Pharmaceuticals Ltd Delhi-110049, India. Rice Bran oil was provided by A.P.Solvex Ltd Dhuri-148024, India. Green Tea was procured from Korangani Green Gold Pvt. Ltd Guhawati- 781005, India.

Chemicals

All the chemicals used in the study were of analytical grade, procured from the credible concerns viz. Sigma, Merck, BDH and Qualigens. Chemicals of higher purity and of scarce availability were obtained from M/S chemical Co; St Louis USA.

Experimental Design

Animals were divided into six groups of six animals each and were treated with ammonium acetate for a period of 45 days: Group-A, (Control); Group-B, (Experimental) Ammonium acetate (AA) treated ; Group-C, Ammonium acetate (AA) treated+ Lycopene (AA-LY); Group-D, Ammonium acetate (AA) treated + Green Tea (AA-GT); Group-E, Ammonium acetate (AA) treated +RBO (AA-RBO); Group-F, Ammonium acetate (AA) treated + Policosanol (AA-PCL). The animals were monitored daily and gain in body weight was recorded twice a week and food consumption was monitored daily. After the completion of feeding schedule, food was withheld and animals were provided only with water, *adlibitum* for overnight.

Induction of Oxidative Stress

Oxidative stress was induced by injecting ammonium acetate intraperitoneally at a dose level of 125mg/kg body weight to the experimental animals.

Biochemical Assays

The blood was withdrawn from retrorbital plexus under mild ether anesthesia for biochemical parameters. Thereafter the animals were sacrificed by cervical decapitation. The liver was excised, washed with ice cold isotonic saline and weighed. A small part of the hepatic tissue was minced and used for enzyme activity assay and other biochemical evaluation. Samples were stored in vials at -25 ^{0}C until further biochemical analysis. All samples were coded prior to analysis.

For enzyme activity assay, 0.8-1.0g of hepatic tissue was minced and homogenized in 10 times its volume of 0.2M/L tris HCl (pH=8.0) containing 0.5M/L $CaCl_2$ using Potter Elevehjem apparatus at 0-4^{0}C using motor driven Teflon

pestle rotated at 3000rpm. The homogenate was centrifuged at 10000x g for 30 minutes at 4^0C and 3/4th of the volume was carefully drawn using Pasteur's pipette. Levels of ammonia (Da Fonseca-Wollheim, 1990) and urea (Varley et al., 1991) were analysed in plasma. Enzyme assay involved, lipid peroxidation (TBARS) (Okhawa et al. 1979), red cell and liver reduced glutathione (GSH) (Sedlak and Lindsey, 1968) and hepatic antioxidant enzymes; glutathione peroxidase (GSHPx) (Necheles et al. 1968), catalase (CAT) (Luck, 1971) and superoxide dismutase (SOD) (Kono, 1978).

STATISTICAL ANALYSIS

Results were expressed as mean ± SEM of 6 rats. Statistical analysis of results was done by Student's-'t' test. The values with $p \leq 0.05$ were considered as statistically significant.

RESULTS

Effect on Nutritional Parameters (Body Weight, Relative Liver Size, Relative Food Consumption)

There was no significant difference in the body weights and relative food consumption in the treatment groups. However, animals of Group-B showed a significant increase in relative liver size as compared to the control ones (Group-A). The treated groups supplemented with neutraceuticals tended to neutralize the effect though the change was not significant ($p \geq 0.05$) as evident from Table 1.

Table 1: Effect of Ammonium Acetate and Neutraceuticals Enriched Diets On Nutritional Parameters of Albino Rats (Values Are Mean ± SEM of 6 Rats in Each Group)

Groups	Body Weight (g)		Relative Liver Size (Liver wt g/100g Body wt)	Relative Food Consumption (g/100g Body wt)
	Initial	Final		
A	179.1±7.70	262.5±17.97	3.1±0.15	7.5±0.54
B	175.8±8.73	302.5±13.76^{NS}	4.1±0.28^{a}	7.6±0.38 ^{NS}
C	179.1±7.70	291.6±10.53^{NS}	3.9±0.24 ^{NS}	7.3±0.28 ^{NS}
D	179.1±7.70	270.8±13.56^{NS}	3.6±0.11 ^{NS}	7.4±0.30 ^{NS}
E	176.6±1.18	286.6±5.13 ^{NS}	3.8±0.13 ^{NS}	7.2±0.22 ^{NS}
F	179.1±7.70	276.6±17.86^{NS}	3.7±0.24 ^{NS}	7.4±0.27 ^{NS}

A- Control	D- Ammonium acetate (AA) +Green tea (AA-GT)
B-Ammonium acetate (AA) treated rats	E- Ammonium acetate (AA) +RBO (AA-RBO)
C- Ammonium acetate +Lycopene (AA-LY)	F- Ammonium acetate (AA) + Policosanol (AA-PCL)

[a] $p < 0.05$ Group B is compared with Group A
[b] $p < 0.05$ Group C; D; E; F is compared with Group B
NS – Non Significant

Effect on Ammonia and Urea Levels

The concentration of ammonia and urea was significantly increased ($p \leq 0.05$) in ammonium acetate-treated group (Table 2). Animals treated with ammonium acetate and neutraceuticals showed significantly lower levels ($p \leq 0.05$) of ammonia and urea as compared to ammonium acetate treated group.

Table 2: Effect of Ammonium Acetate and Neutraceuticals Enriched Diets on Ammonia and Urea Levels of Albino Rats (Values Are Mean ± Sem of 6 Rats in Each Group)

Groups	NH_3 (µmol/l)	Urea (mg/ml)
A	88.2±7.82	10.6±0.65
B	331.1±7.04[a]	22.6±1.19[a]
C	166.7±6.86[b]	13.2±1.04[b]
D	165.9±7.25[b]	11.6±0.96[b]
E	172.4±7.87[b]	12.6±0.78[b]
F	168.4±6.87[b]	11.9±0.73[b]

A- Control	D- Ammonium acetate (AA) +Green tea (AA-GT)
B-Ammonium acetate (AA) treated rats	E- Ammonium acetate (AA) +RBO (AA-RBO)
C- Ammonium acetate +Lycopene (AA-LY)	F- Ammonium acetate (AA) + Policosanol (AA-PCL)

[a] $p < 0.05$ Group B is compared with Group A
[b] $p < 0.05$ Group C; D; E; F is compared with Group B
NS – Non Significant

OXIDATIVE STRESS STATUS

Blood Oxidative Stress Status

Peroxidation potential (TBARS); an index of lipid peroxidation activity significantly increased in Group-B. Inclusion of neutraceuticals to diets reversed the effect. Mean red cell reduced glutathione decreased significantly ($p \leq 0.05$) in experimental group (Group-B). Consumption of different neutraceuticals tended to reverse the effect and significantly increased ($p \leq 0.05$) the values. Mean uric acid level showed a significant increase ($p \leq 0.05$) in Group-B than those of its normal counter parts. The incorporation of neutraceuticals tended to lower the levels as evident from the Table 3.

Table 3: Effect of Ammonium Acetate and Neutraceuticals Enriched Diets on the Blood Oxidative Stress Status of Albino Rats (Values Are Mean ± Sem of 6 Rats in Each Group)

Groups	Peroxidation Potential (nM of TBARS/100ml)	Red Cell Reduced Glutathione (mM/100ml)	Uric Acid (nM/100ml)
A	26.3±0.27	45.8±0.27	10.0±0.30
B	43.0±0.28[a]	37.8±0.57[a]	20.0±0.90[a]
C	36.9±0.25[a]	42.9±0.21[a]	20.0±0.40[a]
D	31.2±0.42[a]	44.1±0.40[a]	10.0±0.60[a]
E	32.3±0.25[a]	43.7±0.29[a]	10.0±0.60[NS]
F	32.3±0.18[a]	42.7±0.25[a]	10.0±0.30[a]

A- Control	D- Ammonium acetate (AA) +Green tea (AA-GT)
B-Ammonium acetate (AA) treated rats	E- Ammonium acetate (AA) +RBO (AA-RBO)
C- Ammonium acetate +Lycopene (AA-LY)	F- Ammonium acetate (AA) + Policosanol (AA-PCL)

[a] $p < 0.05$ Group B is compared with Group A
[b] $p < 0.05$ Group C; D; E; F is compared with Group B
NS – Non Significant

Hepatic Oxidative Stress Status

The results of hepatic mean TBARS concentration showed an increasing trend among the experimental group (Group-B) as compared to the control group. On the other hand, an increase in hepatic mean TBARS levels were lowered by incorporation of neutraceuticals to diets. There was significant reduction ($p \leq 0.05$) in hepatic, mean reduced glutathione levels in Group-B animals as compared to the control group. Supplementation of neutraceuticals reversed the trend and tended to neutralize the effect of ammonium acetate as evident from Table 4.

Table 4: Effect of Ammonium Acetate and Neutraceuticals Enriched Diets on the Hepatic Oxidative Stress Status of Albino Rats (Values are Mean ± Sem of 6 Rats in Each Group)

Groups	TBARS (nM/mg Protein)	Reduced Glutathione (mM/100g)
A	0.76±0.038	384.5±3.56
B	0.87±0.044^{NS}	273.0±3.19^{a}
C	0.79±0.016^{NS}	341.2±4.75^{b}
D	0.75±0.018^{b}	333.4±5.47^{b}
E	0.80±0.001^{NS}	338.4±5.31^{b}
F	0.75±0.021^{b}	342.3±5.10^{b}

A- Control	D- Ammonium acetate (AA) +Green tea (AA-GT)
B-Ammonium acetate (AA) treated rats	E- Ammonium acetate (AA) +RBO (AA-RBO)
C- Ammonium acetate +Lycopene (AA-LY)	F- Ammonium acetate (AA) + Policosanol (AA-PCL)

^{a} $p < 0.05$ Group B is compared with Group A
^{b} $p < 0.05$ Group C; D; E; F is compared with Group B
NS – Non Significant

Antioxidant Enzymes

HFHC diets registered a decreasing trend in the activities of antioxidant enzymes in experimental animals as compared to their control counter parts as evident from the Table 5.

Table 5: Effect of Ammonium Acetate and Neutraceuticals Enriched Diets on Hepatic Antioxidant Enzymes Activity of Albino Rats (Values Are Mean ± Sem of 6 Rats In Each Group)

Groups	Glutathione Peroxidase (g of GSH Utilized/min/mg Protein)	Catalase (Values x 10^{-3} Units/ mg Protein)	Superoxide Dismutase (units/mg Protein)
A	8.6±2.73	56.9±0.54	2.8±0.08
B	6.3±3.09^{a}	46.8±0.48^{a}	1.7±0.11^{a}
C	7.4±1.19^{b}	52.8±0.39^{b}	3.0±0.09^{b}
D	7.4±2.85^{b}	55.4±1.27^{b}	2.8±0.14^{b}
E	8.4±3.09^{b}	51.8±0.62^{b}	3.2±0.06^{b}
F	7.8±2.87^{b}	54.5±0.90^{b}	2.9±0.04^{b}

A- Control	D- Ammonium acetate (AA) +Green tea (AA-GT)
B-Ammonium acetate (AA) treated rats	E- Ammonium acetate (AA) +RBO (AA-RBO)
C- Ammonium acetate +Lycopene (AA-LY)	F- Ammonium acetate (AA) + Policosanol (AA-PCL)

^{a} $p < 0.05$ Group B is compared with Group A
^{b} $p < 0.05$ Group C; D; E; F is compared with Group B
NS – Non Significant

Serum Transaminases

The mean values for transaminases activity are given in Table 6 as biochemical parameters of damage in liver function. Induction of ammonium acetate showed a significant increase ($p \leq 0.05$) in the mean SGPT activity as compared to control group. Supplementation of neutraceuticals enriched diets to animals lowered the mean SGPT activity. There was a decreasing trend, though insignificant ($p \geq 0.05$), in mean SGOT activity of Group-B animals as compared to their control counter parts.

Table 6: Effect of Ammonium Acetate and Neutraceuticals Enriched Diets on Serum Transaminases Activities of Albino Rats (Values Are Mean ± SEM of 6 Rats in Each Group)

Groups	SGPT (IU/L)	SGOT (IU/L)
A	10.6±0.30	7.3±0.11
B	12.2±0.38[a]	7.0±0.14[NS]
C	11.0±0.43[NS]	6.9±0.20[NS]
D	10.9±0.31[b]	6.9±0.19[NS]
E	11.7±0.28[NS]	6.9±0.21[NS]
F	11.2±0.36[NS]	6.4±0.16[b]

A- Control	D- Ammonium acetate (AA) +Green tea (AA-GT)
B-Ammonium acetate (AA) treated rats	E- Ammonium acetate (AA) +RBO (AA-RBO)
C- Ammonium acetate +Lycopene (AA-LY)	F- Ammonium acetate (AA) + Policosanol (AA-PCL)

[a] $p < 0.05$ Group B is compared with Group A
[b] $p < 0.05$ Group C; D; E; F is compared with Group B
NS – Non Significant

DISCUSSIONS

There is no easy "fix" to protect or intervene against diseases associated with exposure to environmental pollutants. Many pollutants, such as heavy metals and persistent organics, bioaccumulate in our bodies, and remediation strategies to remove these chemicals from the environment are extremely difficult and costly. Furthermore, many environmental pollutants induce signaling pathways that respond to oxidative stress; these same pathways are associated with the etiology and early pathology of many chronic diseases (Hennig et al. 2005). Therefore, strategies that modulate the effect of toxicants on pathophysiologic processes involved in disease etiology and progression will be of public health importance.

Environmental toxicants are an underlying denominator of many diet-related chronic diseases, including cardiovascular disease, diabetes, arthritis, osteoporosis, and cancer. There is evidence that various nutrients and phytochemicals are associated with a reduced risk of these diseases by affecting underlying molecular mechanisms (Horia and Watkins 2005; Muñoz-Espada and Watkins 2006; Shen et al. 2004). A primary focus of investigation is needed in developing a better understanding of the bioavailability and bioactivity of flavonoids and carotenoids (Manach et al. 2004) to advance the knowledge of diet and foods to alleviate the damaging effects of environmental pollutants (Hennig et al. 2002). The prevalence of environmental toxicants such as heavy metals and organics that contribute to diminished levels of antioxidants that will aggravate the disease state when dietary intakes of antioxidants are marginal. Evolving studies, derived from epidemiologic and basic research and clinical data, suggest that diet or nutrition, as well as lifestyle changes, can modify pathologies of chronic diseases, as well as diseases associated with environmental toxic insults.

Nutritional intervention has been shown to result in demonstrable improvements in health by lowering the toxicant burden of animals and humans. Given the rich experimental information on the relationship between reactive oxygen species (ROS) and dietary antioxidants as it relates to human health, there is strong evidence suggesting that bioactive food components can be introduced for prevention and intervention purposes at points of disease initiation and/or progression of pathways leading to an unhealthy or lethal phenotype (Seifried et al. 2003).

In the liver, ammonia is removed either in the form of urea in periportal hepatocytes and/or as glutamine in perivenous hepatocytes (Nelson and Cox, 2000). Elevated levels of ammonia and urea in ammonium acetate- treated rats may be due to the liver damage caused by ammonia-induced free radical generation. Reports have shown that excess ammonia induces nitric oxide synthase which leads to enhanced production of nitric oxide, leading to oxidative stress and liver damage (Kosenko et al. 2000; Schliess et al. 2002). The decrease in urea and ammonia in neutraceuticals treated rats may be due to the antioxidant potential of bioactive components which act as free radical scavenger.

Ammonia intoxication enhances lipid peroxidation and leads to the formation of free radicals (Kosenko et al. 1997; Vidhya et al. 2003). This might account for the increased levels of TBARS levels in ammonium acetate-treated rats. Ammonia intoxication depletes the level of glutathione (GSH) (Kosenko et al. 1999).

Aspartate and alanine aminotransferases are sensitive indicators of liver cell injury (Wroblewski, 1959). Enhanced activities of ALT and AST in ammonium acetate-treated rats might be related to the devastation of the liver tissue (Shimamoto et al. 2001, Wroblewski, 1959). Neutraceuticals abolishes ammonia effects, by inhibiting the generation of free radicals and stimulating the glutathione peroxidase (GSHPx), and superoxide dismutase (SOD), thereby decreasing oxidative stress and tissue damage. In our study, activities of antioxidant enzymes (SOD, GSHPx and CAT) decreased significantly in ammonium acetate-treated group. It can be concluded that neutraceuticals could control the oxidative abuse by directly scavenging a variety of radicals and reactive oxygen species and inducing antioxidative enzymes which reduce steady state levels of reactive oxygen species and stabilizing cell membranes which assist them in reducing oxidative damage.

Unprecedented opportunities exist for the expanded use of nutrition to reduce the risk of disease, and these new enabling technologies would be invaluable in that regard.

CONCLUSIONS

Nutrition may be the most reasonable means to develop intervention and prevention strategies for diseases associated with many environmental toxic insults. Many disease indicators, such as inflammation and oxidative stress, are known to be influenced by both nutrition and environmental toxicants. This necessitates the intake of bioactive metabolites having antioxidative potential in appreciable amounts to fight against the toxic effects of environmental pollutants.nutrition as apreventive therapy against disease development.

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